Nucleotide excision repair deficiency is intrinsic in sporadic stage I breast cancer

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The molecular etiology of breast cancer has proven to be remarkably complex. Most individual oncogenes are disregulated in only approximately 30% of breast tumors, indicating that either very few molecular alterations are common to the majority of breast cancers, or that they have not yet been identified. In striking contrast, we now show that 19 of 19 stage I breast tumors tested with the functional unscheduled DNA synthesis assay exhibited a significant deficiency of DNA nucleotide excision repair (NER) capacity relative to normal epithelial tissue from disease-free controls ($n = 23$). Loss of DNA repair capacity, including the complex, damage-comprehensive NER pathway, results in genomic instability, a hallmark of carcinogenesis. By microarray analysis, mRNA expression levels for 20 canonical NER genes were reduced in representative tumor samples versus normal. Significant reductions were observed in 19 of these genes analyzed by the more sensitive method of RNase protection. These results were confirmed at the protein level for five NER gene products. Taken together, these data suggest that NER deficiency may play an important role in the etiology of sporadic breast cancer, and that early-stage breast cancer may be intrinsically susceptible to genotoxic chemotherapeutic agents, such as *cis*-platinum, whose damage is remediated by NER. In addition, reduced NER capacity, or reduced expression of NER genes, could provide a basis for the development of biomarkers for the identification of tumorigenic breast epithelium.

human primary breast cell explant culture | human breast tumor explant culture | breast epithelial attached epispher | hypermutability

Genomic instability is a hallmark of all human cancers (1). It has been hypothesized that genomic instability must arise during carcinogenesis; otherwise the multistep mutational process could not be completed within a human lifetime (2). The only established mechanism of genetic instability is loss of fidelity of DNA replication and/or repair (3). Nucleotide excision repair (NER; also known as "long patch" repair) is a complex pathway that remediates any type of damage that destabilizes the DNA helix (4). Loss of NER is the basis of the hereditary disease xeroderma pigmentosum (XP) (5), which is characterized by a 2,000-fold increase in the incidence of UV-associated skin cancer (6). Diagnostically, loss of NER in patients with XP is clinically established by functional analysis of "unscheduled" (or repair) DNA synthesis (UDS) (7).

Polymorphisms in NER genes have been studied for effects on skin and other types of cancers (8), and an association has been found with the incidence of squamous cell carcinoma (9). Loss of NER capacity has been demonstrated in testicular cancer (10), where it may have important implications for susceptibility to *cis*-platinum-based chemotherapy (11). NER gene polymorphisms have been associated with response to *cis*-platinum in head and neck (12), lung (13), and breast cancer (14). Constitutively low NER levels have been reported in the peripheral blood lymphocytes (PBLs) of patients with breast cancer (15, 16) and their relatives (17). Sensitivity to the clastogenicity of the tobacco smoke carcinogen benzo[*a*]pyrene, a bulky DNA adducting agent whose lesions are remediated by the NER pathway, has also been shown in PBLs from patients with breast cancer (18, 19), and removal of these adducts is impaired in lymphoblastoid cell lines derived from patients with breast cancer (20, 21). There is also evidence that the breast cancer predisposition gene, *BRCA1*, can regulate NER gene expression and repair capacity (22, 23).

Breast cancer is the most common type of cancer among women in the United States and the second leading cause of cancer death (24). Early detection methods, including mammographic screening, have downstaged breast cancer to the point that stage I breast carcinomas (invasive, but 2 cm or smaller and nonmetastatic) (25) are now the most commonly diagnosed form of the disease in the United States. Little is known about such early-stage disease, as there are no representative established cell lines of this tumor type. Most breast cancer studies have historically been performed on a limited number of stage IV pleural effusion-based cell lines (26). Risk factors for breast cancer include the quintessential genotoxic agent ionizing radiation (27) and evidence has also been accumulating implicating tobacco smoke (28).

Breast cancer is a heterogeneous disease by molecular and histological criteria (29–31). To best investigate the potential role of NER in the etiology of breast cancer, we chose to restrict our study to stage I breast carcinomas, which have undergone the least progression after transformation. The distinction between cancer etiology and progression is often blurred in the preclinical cancer literature.

Most studies of DNA repair and cancer have been performed in PBLs (15–17) or in established cell lines derived from metastatic disease (10, 21, 22), especially the HeLa cell line, which exhibits a level of NER at the upper end of the considerably variable range of human cells (32). In vitro cancer studies rarely contain tissue-matched controls as a result of the inability to grow such cells in traditional culture systems. Based on tissue engineering techniques, we have developed a reliable and unique method to culture breast epithelial cells (33, 34). This system allows for direct analysis of the somatic changes occurring during breast oncogenesis, rather than relying on a surrogate tissue, that may be affected by tissue-specific differences in DNA repair (35, 36). Through functional analysis of such primary explants, we now report a ubiquitous deficiency of NER activity in a series of sporadic stage I breast

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tumors, suggesting that loss of NER capacity is an intrinsic element in the development of breast neoplasia.

Results

Selection and Culture of Tumor Samples. The most common subtype of breast tumors is invasive ductal carcinoma, accounting for more than 80% of incident breast cancers. Within this group, stage I tumors (TSIs) account for the greatest proportion of diagnosed cases. Among invasive tumors that may progress to higher stages, these have undergone the least progression beyond the initial transformation, and so should be the most informative for studying breast cancer etiology. TSIs, by definition, as they are so small, present a challenge to the pathologist, who would prefer to examine the entire tumor mass to ensure that there are no heteromorphic areas that would change the initial diagnosis. Thus, TSIs are less likely to be released for research purposes than larger tumors. We fractionated the samples we obtained, freezing a portion and embedding a second segment for confirmatory pathological analysis. Within this group of tissue samples, our rate of successful culturing for UDS analysis was 85%.

Tissue samples from 19 clinically stage I invasive breast ductal carcinomas, histopathologically confirmed to be free of adjacent normal breast epithelium, were processed for UDS analysis. In our culture system, normal breast epithelium forms 3D clusters called attached "epispheres," consisting of 30 to 100 rounded epithelial cells (36) (Fig. 1*A*). In contrast, tumor cells under the same culture conditions lack the ability to form these structures, assume a more mesenchymal appearance (Fig. 1*B*), and tend to grow as autonomously motile singleton cells. The transformed phenotype of tumor cells in culture was confirmed using parameters applied in clinical analysis of fine needle aspiration biopsies: lack of cohesiveness, increased nuclear to cytoplasmic ratio and the presence of prominent nucleoli (Fig. 1*B*).

The clinical features of our population of patients with breast cancer are summarized in Table 1. None of our patients reported a significant family history of breast or related cancers. The patients ranged in age from 30 to 82 y, including seven premenopausal and 12 peri- or postmenopausal, with an average follow-up of 8.34 y. None of these patients received presurgical chemotherapy or radiation therapy. The tumors ranged in size from 1.2 to 2.0 cm, and included grades 1 to 3. With regard to estrogen receptor (ER) and progesterone receptor (PR) status, nine tumors were ER^+/PR^+, one was ER^+/PR^-, two ER^-/PR^+, two ER^-/PR^-, three overexpressed both ER and PR, and one each overexpressed ER or PR. In three patients, the tumors have subsequently recurred, one as ductal carcinoma in situ, one as both ductal and lobular carcinoma in situ, and one as invasive ductal carcinoma. Negative lymph node status was confirmed in 13 cases by examination of six to 39 axillary lymph nodes, and in one case by examination of both the sentinel node and four axillary nodes. Five patients declined axillary excision, so lymph node status could not be established. This group was not significantly different from the confirmed lymph node-negative population with regard to tumor size, nuclear grade, hormone receptor status, or tumor S-phase index, but they were somewhat older (mean age, 69.6 y vs. 57.3 y; $P = 0.053$). Two of these patients have subsequently died, one from heart disease and one from a recurrence of breast cancer (the previously described invasive-carcinoma).

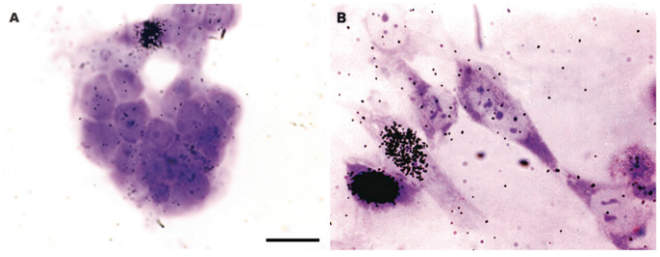

Fig. 1. Primary nondiseased epithelium and tumor cultures. Photomicrographs (1,000× bright-field magnification) of primary epithelial cultures of (*A*) normal breast reduction epithelial cells showing typical attached episphere architecture and (*B*) stage I breast tumor cells after processing for the UDS assay. Tumor cells exhibit classical higher nuclear-to-cytoplasmic ratios, lack of cohesiveness and epithelial architecture, as well as more activated, more prevalent nucleoli. Nuclei with dense silver grains are in S-phase (i.e., "scheduled" DNA synthesis), whereas the much lighter labeling of other nuclei is a result of label incorporation during repair (i.e., UDS) synthesis. (Scale bar in *A*: 4 μm.)

NER Capacities of TSIs. Unlike simple polymorphism studies, the UDS assay provides a quantitative functional assessment of NER capacity. We have previously reported the UDS capacities of 23 samples of breast tissue derived from breast reduction mammoplasties (34, 37). These results, from the breast reduction epithelium (BRE) of women who were disease-free with regard to breast cancer, represent the "normal" level and range of NER in this tissue in the population. Fig. 2 shows the NER capacity measured in breast tumor and normal primary cultures expressed relative to the mean of these normal BRE. The mean NER capacity of the TSI samples was significantly lower than that of BRE, averaging only 44% of normal activity ($P < 0.001$). Multivariate and pairwise analyses revealed no association between tumor NER capacity and patient age at diagnosis, menopausal status, tumor size, nuclear grade, ER and/or PR status, sample cell proliferation (as measured by the S-phase index), recurrence, or disease-free interval (Fig. 3). Statistical associations observed within this data set included positive correlations between ER and PR status ($P = 0.001$) and patient age and recurrence ($P = 0.031$) and inverse correlations between patient age and tumor grade ($P = 0.001$) and patient age and disease-free interval ($P = 0.013$), all consistent with previous studies.

Microarray Analysis. Three representative cultures each of BRE (from one premenopausal donor and two postmenopausal) and TSI (one premenopausal onset, two postmenopausal onset, two ER^+, one ER^-) were successfully expanded for molecular analysis. RNA hybridization from these expanded explant cultures distinguished and grouped the normal and tumor-derived samples based on expression of a set of 676 probe sets associated with genomic instability genes (Fig. S1).

Twenty canonical NER genes were identified among the 38,500 known transcripts on the Affymetrix U133 2.0 Plus chip. All these genes are essential for NER, although some are also required for such fundamental metabolic processes as transcription, replication, and cell cycle regulation (38, 39). All 20 of these core NER genes were expressed at lower levels in the tumor relative to the breast reduction samples (Fig. 4*A*). Although there is a general trend toward gene underexpression in the tumor among all genes in the analysis (59.5% of total probe sets), a significantly greater proportion of NER genes exhibit reduced tumor expression ($P = 0.0002$).

RNase Protection Analysis. Independent validation of the mRNA expression levels for these NER genes was provided by quantitative RNase protection analysis (RPA; Fig. 4*B*). One highly expanded BRE and one similarly expanded TSI sample, previously analyzed by gene expression microarray (Fig. S2), were reanalyzed by this method (Fig. S3 *A* and *B*). Expression levels measured by this method were highly correlated with those previously measured by microarray ($P = 0.002$), with 19 NER genes showing significantly lower expression in the tumor versus normal [$P < 0.001$ for all genes except *RPAp14* ($P = 0.026$)]. The last gene, *ERCC1*, actually showed a significant increase in mRNA expression in this limited analysis ($P = 0.030$).

Western Analysis. In the simplest model, the low levels of steady-state mRNA observed for many NER genes in the TSI cultures by microarray and RPA would result in correspondingly low levels of their protein products, causing the observed deficiency in NER capacity in these cells. Validated monoclonal antibodies were obtained for the products of five NER genes, including four

Table 1. Clinical characteristics of the patient population

Patient no.	Age, y	Tumor size, cm	Tumor grade	Tumor ER status	Tumor PR status	Axillary lymph nodes examined	Tumor S-phase index, %	Tumor NER capacity*	Known disease-free interval, y
1	70.6	1.2	2	Positive	Positive	15	0.0	0.30	7.9
2	80.2	1.5	1	Positive	Negative	0	14.3	0.01	7.4
3	57.3	1.3	3	Negative	Negative	9	6.0	0.55	7.7
4	64.8	1.5	2	Positive	Positive	9	10.2	0.28	9.9
5	39.2	1.6	3	Negative	Positive	9	15.5	0.26	7.1
6	42.2	1.7	3	Overexpressed	Overexpressed	19	21.9	0.53	12.7
7	34.7	1.5	3	Negative	Positive	39	3.2	0.28	12.0
8	63.3	1.7	2	Overexpressed	Negative	21	17.4	0.23	6.5
9	52.4	2.0	2	Positive	Positive	20	27.7	0.63	11.7
10	53.6	1.5	3	Overexpressed	Overexpressed	17	2.1	0.48	11.4
11	45.4	1.2	3	Positive	Positive	0	26.1	0.74	10.9
12	72.8	1.2	1	Overexpressed	Overexpressed	0	34.7	0.44	8.7
13	68.3	1.3	3	Positive	Positive	0	26.9	0.37	6.7†
14	29.9	1.2	3	Negative	Negative	19	2.3	0.69	10.7
15	36.7	1.6	3	Positive	Positive	21	2.8	0.30	10.2
16	81.5	1.5	2	Positive	Positive	0	0.2	0.44	4.9‡
17	44.1	1.2	2	Positive	Overexpressed	10	23.7	0.52	9.4
18	70.6	2.0	2	Positive	Positive	1 + 4§	3.8	0.60	10.0
19	56.9	1.3	2	Positive	Positive	6	24.0	0.68	3.1

*Relative to mean of 23 normal breast epithelial samples derived from reduction mammoplasties (33,36).
†Time to death unrelated to cancer.
‡Time to recurrence leading to subsequent death.
§One sentinel node and four axillary lymph nodes.

genes shown to be consistently underexpressed in stage I breast tumors by both microarray and RPA, as well as for the product of the *ERCC1* gene, which exhibited variable results at the mRNA level. These gene products were quantified from representative breast reduction and tumor extended explant cultures (Fig. S3 *C* and *D* and Fig. 4*C*). Overall, protein levels were highly concordant with the mRNA expression levels as determined by microarray ($P < 0.001$) or RPA ($P = 0.007$). Significantly lower levels of XPA and CSB proteins (both $P < 0.001$) were observed in the tumor, as well as DDB2 (XPE) protein ($P = 0.004$). The lower protein amount for DDB1 in the tumor failed to reach statistical significance ($P = 0.45$), as did the slight increase in ERCC1 protein ($P = 0.79$). *ERCC1* was the only NER gene to show somewhat inconsistent overexpression at both the mRNA and protein level. This apparent lack of consistent down-regulation of *ERCC1* may be specific to this gene, as down-regulation of its partner protein XPF would be sufficient to lower its activity. With the exception of a single, severely affected individual (40), the null phenotype for *ERCC1* is not found in human XP disease states, so the expression of this gene below a certain level may not be generally viable (5).

Discussion

Historically, NER has been associated almost exclusively with the repair of UV-induced DNA damage, primarily because of the phenotype of patients with the NER-deficiency disease XP (41). Recent work showing the importance of NER in the repair of bulky DNA adducts, such as those caused by tobacco smoke (42) and intrastrand cross-links caused by the chemotherapeutic agent *cis*-platinum (43) have generally been interpreted very narrowly. We, however, have long believed that the generality of the nature of the DNA lesions that induce NER—those that distort the DNA helix or impede gene transcription—imply that NER is the major human pathway for generalized base damage repair (44), especially for the repair of damage caused by common environmental carcinogens (45). The relative size and complexity of the NER pathway should make it a frequent target of mutational or epigenetic alteration, a hypothesis supported by

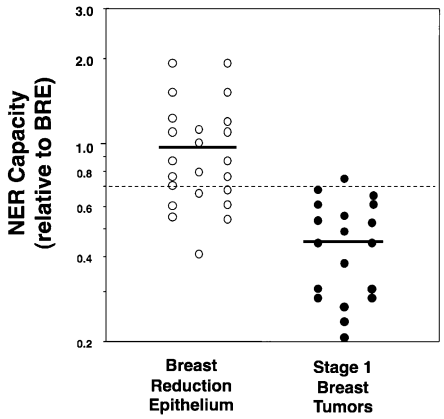

Fig. 2. NER capacity of BRE and stage I breast tumors expressed relative to average BRE. Comparison of NER capacities of primary explant cultures established from breast reduction mammoplasty tissue from nondiseased controls ($n = 23$) and stage I ductal carcinoma ($n = 19$). Means for each population are indicated by the horizontal line. The cutoff used for evaluation calculating an odds ratio (*Discussion*) is indicated by the dotted line.

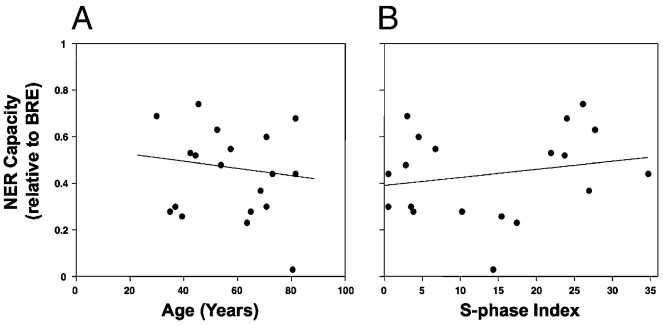

Fig. 3. Lack of effect of donor age and in vitro proliferation rate on NER capacity in stage I breast tumors ($n = 19$). Linear regression of NER capacity with (*A*) patient age and (*B*) S-phase index (i.e., percentage of cells in S-phase), showing no significant associations: age ($P = 0.42$), proliferation rate ($P = 0.38$). We have previously shown that these factors show no association with NER capacity in the normal breast epithelium (33, 36).

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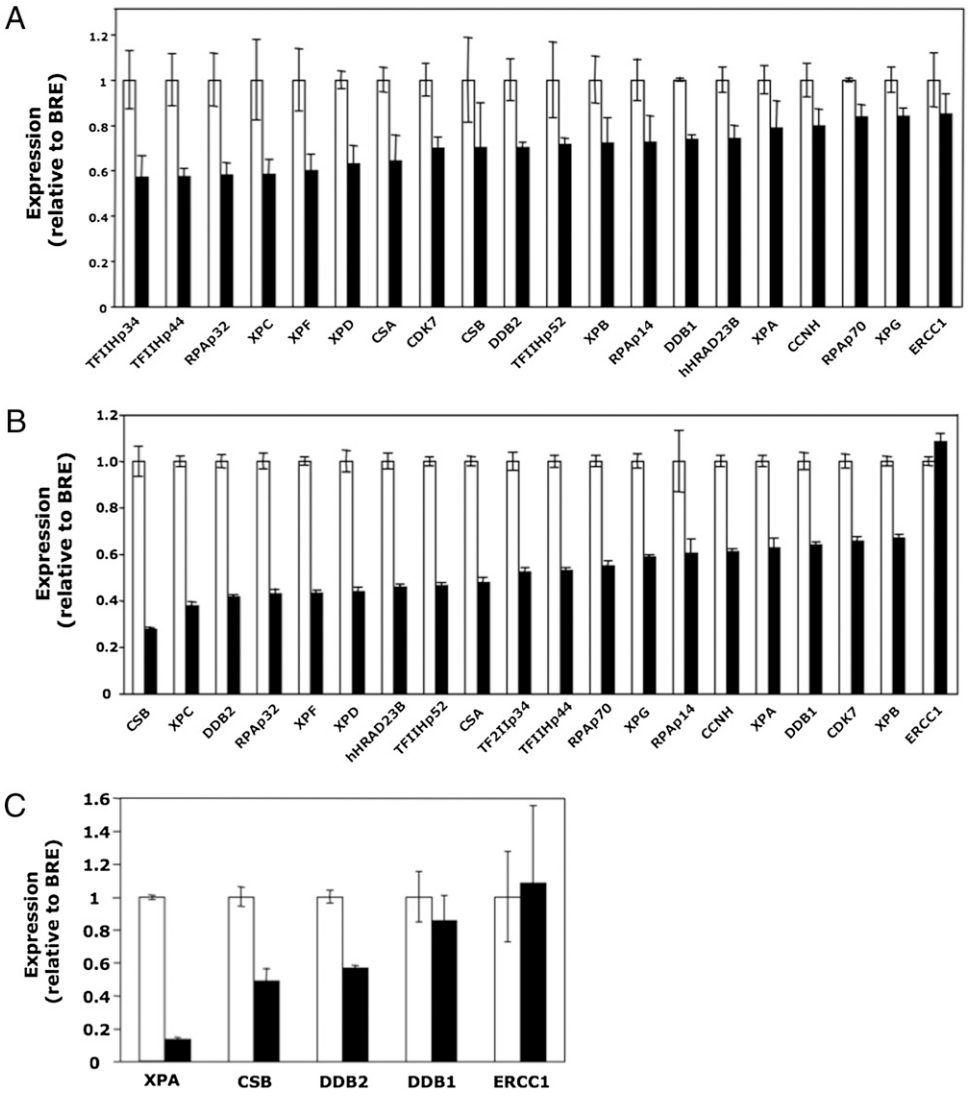

Fig. 4. Molecular analysis of NER in nondiseased BRE and breast TSI explant cultures. (*A*) Microarray analysis of NER using the Affymetrix U133 2.0 Plus chip processed using the Probe Logarithmic Intensity Error algorithm. Summary of results for 20 NER genes shows reduced mRNA expression in tumor (solid bars, $n = 3$) versus age-matched normal breast epithelium (open bars, $n = 3$). The observed reduced expression in TSI expression was individually significant for nine genes: *TFIIHp44* ($P = 0.024$), *RPAp32* ($P = 0.031$), *XPD* ($P = 0.013$), *CSA* ($P = 0.046$), *CDK7* ($P = 0.026$), *XPE* ($P = 0.035$), *DDB1* ($P = 0.0002$), *hHRAD23B* ($P = 0.032$), and *RPAp70* ($P = 0.036$); and came close to significance for four others: *TFIIHp34* ($P = 0.054$), *XPC* ($P = 0.093$), *XPF* ($P = 0.060$), and *XPG* ($P = 0.070$). (*B*) Quantitative RPA of 20 NER genes in nondiseased BRE and breast TSI explant cultures. Gene expression for all genes except *ERCC1* is significantly lower ($P < 0.05$) in breast tumor (solid bars) than in normal breast epithelium (open bars). Data given and variability are derived from nine to 14 individual analyses for each gene. (*C*) Western analysis analyses of five NER proteins in nondiseased BRE and breast TSI explant cultures. Protein levels were reduced for the *XPA*, *CSB*, *DDB1*, and *DDB2* gene products in breast tumor (solid bars) relative to normal breast epithelium (open bars). These reductions were significant for the XPA, CSB, and DDB2 proteins as detailed in the text. Data given and variability are derived from three replicate analyses for each protein.

recent evidence from Castro et al. (46). Our observation that the majority of NER genes are down-regulated in early-stage breast cancer would argue for an epigenetic mechanism; however, whether such epigenetic down-regulation is secondary to mutation at a known NER structural gene or an unknown NER regulatory gene is not clear.

Patients with XP generally manifest cancers in the skin and other tissues exposed to UV, although internal cancers do occur (6), and their incidence may be limited by the early mortality associated with the disease, as has been observed for other tumor types arising in survivors of childhood retinoblastoma (47). Transgenic mice with disruption of NER genes exhibit high mutation frequencies in multiple tissues (48) and manifest internal tumors (49). Loss of NER constitutes an underlying mechanism of genomic instability (3), a fundamental process of carcinogenesis (2), and our results with early-stage breast tumors suggest it is intrinsic to the etiology of breast cancer.

A number of investigators have demonstrated an association between breast cancer incidence and NER deficiency, all by using PBLs (15–21). Our ability to reliably culture epithelial tissue has allowed us to extend this observation to the breast itself, including the concurrent analysis of nondiseased breast epithelial tissue. The PBL-based data would suggest that NER deficiency is a preexisting condition in these patients. None of our patients had XP itself or a significant family history of cancer, however. Moreover, UDS analysis of tissue samples from the contralateral breast of two patients with breast cancer, one a *BRCA1* mutation carrier, revealed normal levels of NER (37). Whether NER deficiency in patients with breast cancer is preexisting, acquired during oncogenesis, or some combination of the two is still to be determined.

Many molecular markers have been investigated for their potential role in breast carcinogenesis and as predictive or prognostic factors. In contrast to our results, however, all but one appear to be present in one third or fewer of all breast tumors (29). The notable exception is inactivation of the DNA damage-inducible gene 14–3-3 σ (50), which may be related to the effects on the NER pathway that we have observed. The highest NER capacity observed in our TSI samples was 74% of normal [NER capacities of 50% of normal and lower are considered to be diagnostic for XP (4, 51)]. If 0.70 times the average of the NER capacity of the breast reduction samples is established as a cutoff (Fig. 2), the sensitivity of detecting tumors based on reduced NER levels alone is 95%, the specificity is 74%, and the odds ratio is 53.8 (95% CI, 28.3–102.4). Thus, measurement of NER capacity in breast cellular material such as would be obtained by needle or core biopsy could support diagnosis of an early-stage tumor. Direct measurement of functional NER activity is presently impractical in such cases, however, and analysis of NER genes at the level of mRNA or protein is complicated by their low baseline expression, nuclear localization and the relatively small (50%) difference in expression we have observed in early stage breast tumors. Such changes in DNA repair capacity can lead to large

changes in susceptibility to DNA damage and mutation, however, which have been exploited as the basis of diagnostic biomarkers (52, 53). Our results, on expanded, purified tumor and normal cultures, are likely to be better than those obtained from patients' samples, which are often limited in size and contaminated by extraneous cell types, such as vascular, immune, and blood cells in the tumor samples. Unlike most in vitro studies, we have the benefit of comparing our tumor analyses versus tissue-matched controls, grown in a system that attempts to minimize "adaptation" to culture.

The NER pathway remediates DNA damage caused by a number of genotoxic cancer chemotherapeutic drugs, including cross-linking agents such as *cis*-platinum (43), as well as bulky DNA adducting agents such as *N*-acetoxy-2-acetylaminofluorene (54). Thus, our NER data suggest that early-stage breast cancer should be particularly vulnerable to such agents. It may be useful to consider these findings in the evaluation of clinical trial data, or in the design of new chemotherapeutic regimens, as has been suggested for testicular cancer (11). These data also support the idea that early-stage tumors can be treated efficaciously by chemotherapy, a practice that has become more common during the past decade.

Materials and Methods

Tissue Samples and Primary Explant Culture. Tissues from stage I invasive breast ductal carcinoma were obtained as samples excised within the tumor margin on all sides. One third of each specimen was used to initiate explant culture, one third was processed for confirmatory histological assessment, and one third was frozen for future studies (Fig. S4). All surgeries were performed at Magee–Womens Hospital (institutional review board approval 0609002). Culture conditions for all specimens were as previously described (34), involving physical disaggregation of the tissue and culture in duplicate (or more) on Matrigel thin-coated two-chamber slides in serum-rich MWRIα primary culture medium (33). ER and PR status were determined clinically by using standard peroxidase antibody staining. Standard inquiries pertaining to family and personal cancer histories were made at the time of diagnosis.

Extended Explant Cultures. Six samples—three stage I breast tumors and three age-matched normal breast reduction tissues—were expanded in culture for the generation of RNA for molecular analysis. The tumor samples were derived from a 52-y-old woman (patient 9 in Table 1) who presented with a 2-cm ER^+/PR^+ tumor with a nuclear grade of 2, a 35-y-old woman (patient 7 in Table 1) who presented with a 1.5-cm ER^-/PR^+ tumor with a nuclear grade of 3, and an 81-y-old woman (patient 16 in Table 1) with a 1.5-cm ER^+/PR^+ tumor with a nuclear grade of 2. These tumor samples had NER capacities of 0.63, 0.28, and 0.44 of normal breast epithelium, respectively. The normal samples were derived from a 62-y-old woman with a body mass index (BMI) of 25.5 ("overweight" [55]), a 34-y-old woman with a BMI of 29.2 ("overweight"), and a 26-y-old woman with a BMI of 35.0 ("obese"). These samples had measured NER values of 1.03, 0.61, and 0.72 of the average capacities of our 23 normal breast specimens, respectively.

Historically, breast explants have had limited growth potential in culture (56), and, with rare exceptions (57), have been unsuitable for molecular analyses. To generate sufficient cells for RNA and protein extraction, confluent normal primary cultures were judiciously trypsinized at room temperature and subcultured onto a fresh Matrigel substratum. These cultures were fed every 2 to 3 d, initially through replacement of half the medium and its replacement with fresh MWRI explant culture medium, and later through replacement of total medium. The tumor primary cultures were similarly handled. Cells (10^6 each) were harvested and extracted for total RNA. We refer to cultures such as these, which have been passaged several times but are not established clonal cell lines, as "extended explants." Two cultures, the first described of the normal and tumor samples above, were successfully further expanded for generation of RNA and protein for RPA and Western analysis.

UDS Assay. NER was measured by using the autoradiographic UDS assay (58). After 7 d without passaging, actively growing primary cultures in one chamber were irradiated with 14 J/m^2 of UV at 254 nm, with the second chamber shielded to act as a control. Cells were then incubated in medium containing 10 μCi/mL [^{3}H]methyl thymidine for 2 h, the label chased with cold thymidine for 2 h, and the labeled cells fixed, dried, and exposed to photographic emulsion for 10 to 14 d. As the UDS assay is a relative measure of label incorporation, two positive controls were included in every experiment: fresh foreskin fibroblasts (FFs) and the breast carcinoma cell lines MCF-7 and MDA-MB-231. After photographic development of slides, the nuclei were stained and silver grains quantified at a magnification of 1,000×. An average of 4.5 slides were scored for each of the stage I breast tumor samples analyzed in this study by an average of three independent counters. An average of 162 nuclei were scored per slide, or almost 750 nuclei per sample, at an average of 12.0 silver grains per nucleus. NER results from the 23 normal explant cultures have been previously reported (34, 37).

Statistical Analysis. The final count from slides of the same cell-type within the same experiment and developed the same day were averaged together and initially expressed relative to concurrently analyzed FF. The possible effects of donor age, S-phase index, and clinical variables such as tumor grade, size, and hormone receptor status on NER capacity were evaluated using linear regression at an α value of lower than 0.05. Age was also evaluated by assignment into two groups, premenopausal (< 50 y old) and peri- or postmenopausal (≥ 50 y old). ER and PR status were separated into three categories: negative, positive, and overexpressed. Quantitative comparisons between tumor and breast reduction properties were performed by using the parametric two-tailed *t* test. Categorical variables were also compared by using the χ^2 test.

Microarray Analysis. Total RNA was isolated using the RNeasy Kit from Qiagen (cat no. 74004). Microarray expression analysis was carried out at the Clinical Genomics Facility of the University of Pittsburgh Cancer Institute by using the U133 2.0 Plus chip (Affymetrix). Before samples were analyzed, RNA integrity was evaluated by using Bioanalyzer technology (Agilent). Biotin-labeled cRNA synthesis, hybridization, washing, staining, and scanning were done following the manufacturer's protocols (Affymetrix). Total mRNA (2 μg) was labeled with fluorescent tags by a PCR and then hybridized to the chip set. Results were captured using a GeneChip Scanner 3000 with GeneChip Operating Software (Affymetrix).

The Probe Logarithmic Intensity Error algorithm (Affymetrix) was used to derive numerical data from the fluorescent signals. All probe set IDs corresponding to the genes of interest were averaged and gene expression reported relative to the average of the three BRE samples analyzed. Dendrograms were created by supervised hierarchical clustering analysis of the Log2-transformed data computed through Agilent GeneSpring GX software.

RNase Protection Assay. The multiprobe RPA kits hNER1 and hNER2 (BD Riboquant; Pharmingen) were used to analyze the mRNA expression of 20 genes in the NER pathway—*XPC*, *hHR23B*, *XPA*, *RPAp70*, *RPAp32*, *RPAp14*, *XPG*, *XPF*, *DDB1*, *DDB2* (hNER1), *XPB*, *XPD*, *TFIIHp34*, *TFIIHp44*, *TFIIHp52*, *CDK7*, *CYCH*, *ERCC1*, *CSA*, and *CSB* (hNER2)—following the manufacturer's directions. Briefly, radiolabeled antisense RNA probes were synthesized using the Pharmingen in vitro transcription kit. Control samples in each experiment included two replicates each of RNAs isolated from the cells used as controls in our UDS analyses: FF, MCF-7, and MDA-MB-231, and yeast tRNA as a negative control. Densitometric quantification was performed against a 1:3 serial dilution of the provided RNA standard, comprised of 2, 0.67, 0.22, and 0.07 μg of human control RNA-2 (Pharmingen). Unhybridized probe was run as a size standard on each gel. Scanning densitometry was used to quantify results from the RPAs by using the Harmony program, version 4.03 (Videk). Normalization of band intensities was performed using the *GAPDH* housekeeping gene.

RPA Data Analysis. At least four independent experiments were run for each sample with each of the two kits used. Three independent harvests of RNA were used. In general, the total results reflect more than 10 experiments (gels) per cell line. The normalized intensities of the bands for each gene were initially expressed relative to the average of the FF control, allowing for comparison across experiments. Finally, gene expression was calculated relative to the average of the three breast reduction samples analyzed. Comparisons between groups were done by using two-tailed *t* tests at an α value of lower than 0.05.

Western Analysis. Total protein was harvested from snap-frozen cell pellets using whole cell lysis in the presence of detergent. Cells were incubated for 30 min on ice in an equivalent volume of extraction buffer. The lysate was cleared of unlysed cells, and the supernatant was collected, snap-frozen, and stored at −80 °C. Protein concentration was quantified using the Bradford protein assay (BioRad).

Western blotting was carried out for the CSB (using monoclonal antibody sc-25370), DDB1 (sc-16292), DDB2 (sc-16295), ERCC1 (sc-53281 or RW018), and XPA (CJ05) proteins (commercial antibodies purchased from Santa Cruz Biotechnology; RW018 and CJ05 were gifts of Richard Wood, The University of Texas M.D. Anderson Cancer Center, Science Park–Research Division, Smithfield, TX).

Total protein (25–50 μg) was resolved using a 4% SDS glycine stacking gel/8% SDS glycine polyacrylamide running gel. Controls on each gel included lysate from FF. Proteins were denatured at 100 °C in the presence of loading buffer. Electrophoresis was performed at 120 V in Tris glycine SDS. The proteins were transferred in Tris glycine methanol buffer to a PVDF membrane (Immobilon-P; Millipore). Membranes were washed in PBS solution with 0.1% Tween or Tris-buffered saline solution with 0.1% Tween and blocked overnight at 4 °C with 5% (sc-25370) or 20% (CJ05) milk in Tris-buffered saline solution/0.1% Tween, 20% milk in PBS solution/0.1% Tween (sc-53281, RW018), or 2% I-block (sc-16292, sc-16295, sc-5546; Applied Biosystems). Visualization for alkaline phosphatase-conjugated secondary antibodies was performed by using the CPD Star assay reagents (Tropix), and for horseradish peroxidase-conjugated secondary antibodies by using the SuperSignal West Femto kit (Pierce). Images were captured on Kodak Biomax XR film and a BioRad Chemidoc system by using the Quantity One software package.

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